RESEARCH ARTICLE

Determination of Genistin Content in Flemingia philippinensis Merr. et Rolfe by High Performance Liquid Chromatography (HPLC)

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Abstract: To set up the HPLC method in determination of the genistin content in Flemingia philippinensis Merr. et Rolfe. Method: Extract and filter the crushed Flemingia philippinensis Merr. et Rolfe that has been soaked in methanol, the filtrate of which is to be taken out as the sample. Shimadzu LC-2010A is adopted in this experiment which is performed on Agilent Extend-RP column with acetonitrile-0.1% phosphoric acid aqueous solution (19:81) as mobile phase. The flow rate is 1.0 mL/min and the UV detective wavelength is 262 nm. Result: The linear relationship is excellent with higher recovery rate within the range. This method is rapid, simple and accurate.

Keywords: Genistin; Flemingia philippinensis Merr. et Rolfe; High Performance Liquid Chromatography (HPLC)

Flemingia philippinensis Merr. et Rolfe is a herb from the dry roots of various plants of Flemingia, which is commonly known as Jack Horse, Qianli Horse, Hanging Horse Column and Dalihuang etc.[1]. According to traditional Chinese medicine, Flemingia philippinensis Merr. et Rolfe tastes sweet, spicy and slightly bitter. It is mild and non-toxic, which is effective in strengthening waist and kidney, removing dampness through diuresis, activating blood and dredging collaterals, eliminating stasis and detoxifying etc.. Flemingia philippinensis Merr. et Rolfe could be used in the treatment of rheumatic pain, lumbar muscle strain, chronic nephritis, bruises, abscess, hemiplegia, impotence, leucorrhea increase etc.[2]. Current, Flemingia philippinensis Merr. et Rolfe has not been included in Chinese Pharmacopoeia. But the appendix of Chinese Pharmacopoeia (2005) has illustrated that the herb is from Flemingia philippinensis Merr. et Rolfe, F. macrophylla (Wind.) Prain and F. ferrugirnea Grah. ex Wall[3]. According to Hunan Chinese herbal standards, the sources of the herb are the same three kinds of plants mentioned above[4]. However, the source of the herb is limited to only Flemingia philippinensis Merr. et Rolfe in Guangdong Provincial Traditional Chinese Medicine criteria and Shanghai Traditional Chinese Medicine criteria[5][6]. And in Guangxi standard Chinese herbal medicines, the sources of the herb are Flemingia philippinensis Merr. et Rolfe and F. macrophylla (Wind.) Prain[7]. All the criteria mentioned above have performed routine identification, tissue identification and preliminary chemical identification of the herb, lacking of specific component identification and content determination. Recently, Flemingia philippinensis Merr. et Rolfe is widely used in Chinese traditional medicine like Fuke Qianjin Pian and Jinji Chongji with an annual demand exceeding 1 million kilograms[8]. Due to the the complicated genera and families of Flemingia philippinensis Merr. et Rolfe, the current quality control items could not effectively distinguish the quality of the herb. As a result, it is urgent to enhance the quality criteria of medicine and involve in content determination and other control items to guarantee the quality of clinical medication. It is reported that the main active component of Flemingia philippinensis Merr. et Rolfe is flavonoids[9]. This study takes the flavonoids-genistin isolated from Flemingia philippinensis Merr. et Rolfe as the marked ingredients to set up a Liquid Chromatography to determine the content of Flemingia philippinensis Merr. et Rolfe from different places and part of the marketed Flemingia philippinensis Merr. et Rolfe. It is aimed to offer a new guidance and idea for the quality evaluation and control of Flemingia philippinensis Merr. et Rolfe.

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1. Instruments and Reagents

Shimadzu LC-2010A HT is adopted with phosphoric acid as analytical reagent and acetonitrile as chromatogram. The genistin is self-made. Flemingia philippinensis Merr. et Rolfe and F. macrophylla (Wind.) Prain are collected in Xinping, Yangbi and Jinghong in Yunnan Province in October 2007. And the marketed herb is purchased at the Chinese herbal medicine market of Chrysanthemum Village in Kunming. The plant samples are identified as Flemingia philippinensis Merr. et Rolfe and F. macrophylla (Wind.) Prain. And the marketed herbs are identified as F. macrophylla (Wind.) Prain. Both the plant samples and marketed herbs are identified by Associated Professor Yang Shude from Teaching and Research Department of Drug Identification of Yunnan University of Traditional Chinese Medicine.

2. Methods

2.1 Chromatographic Conditions

This experiment is performed on Agilent Extend-ODS column (5μm, 4.6 mm×250 mm) with acetonitrile-0.1% phosphoric acid aqueous solution (19:81) as mobile phase for elution. The detective wavelength is 262 nm, the flow rate is 1.0 mL/min, the column temperature is 30℃ and the sample size is 10μL.

2.2 Preparation of Reference Substance

2.2.1 Preparation of Reference Substance

Crush 5 kg of Flemingia macrophylla (Wind.) Prain and soak it into methanol for three times with each time adding another 40kg methanol for 48h. Decompress and recover the methanol extract to get 0.61kg extract, putting 3kg water into which to suspend and filter with macroporous resin. Elute with water first to remove monosaccharides and other impurities and then elute for the second time. Decompress and recover the solution and mix the extract with 0.6kg silica gel. Dry and elute the solution with chloroform-methanol (10:1) to (3:1) and separate the extract with 4.5kg silica gel column to get five parts. Among, elute the third part with trichloromethane-methanol (5:1) and separate the extract to merge the main spotted flow. Concentrate, dissolve in proper amount of methanol, place, crystallize, filter and dry the extract to get 0.36g white powder-like crystal (compound 1), which is identified as genistin.

2.2.2 Structure Identification of Reference Substance

Compound1: white powder-like. UV λ^{MeOH}_{max}(nm): 262, 327; FABF minus-MS(m/z): 431[M-H]$^-$; ^{1}H-NRM(500MHz, CD_3OD)δ: 12.93(1H, s, 5-OH), 9.65(1H, s, 4′-OH), 8.40(1H, s, 2-H), 7.38(2H, d, J=8.4Hz, 2′, 6′-H); 6.81(2H, d, J=8.4Hz, 3′, 5′-H), 6.70(1H, d, J=1.8Hz, 8-H),6.41(1H, d, J=1.8Hz, 6-H), 5.45(1H, d, J=1.8Hz, 1″-H), 3.12-5.17 (m, sugar protons); ^{13}C-NRM(500MHz, CD_3OD)δ: 154.6(C-2), 122.6(C-3), 180.6(C-4), 161.7(C-5), 99.6(C-6), 163.0(C-7), 94.6(C-8), 157.5(C-9), 106.1(C-10), 121.1(C-1′), 130.2(C-2′,6′), 115.2(C-C-3′,5′), 157.3(C-4′), 99.9(C-1″), 73.1(C-2″), 77.2(C-3″), 69.7(C-4″), 76.4(C-5″), 60.7(C-6″). The above mentioned spectral data is in line with the reported data of genistin[3]. Therefore, this sample could be identified as genistin.

2.3 Preparation of Solutions

2.3.1 Preparation of Reference Solution

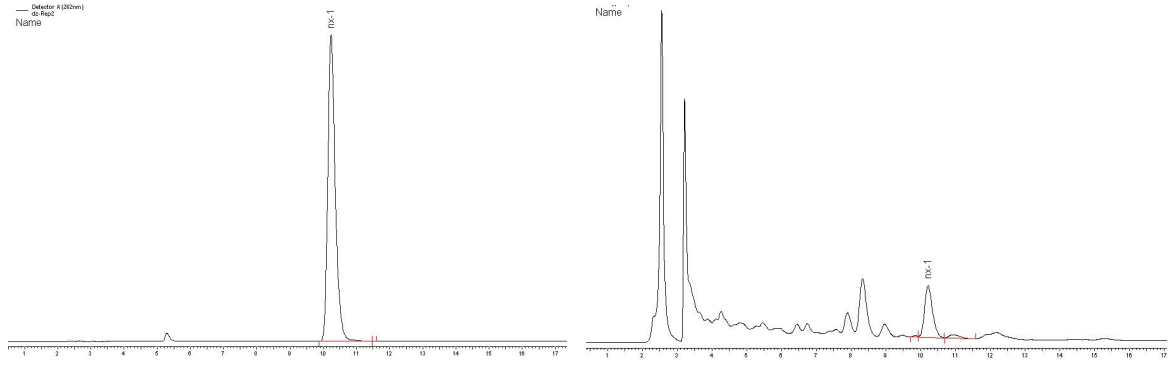
Genistein is precisely weighed and mixed with methanol to prepare 0.0211 mg/mL reference solution. After dilution, the concentration of genistein is respectively as follows: 0.0000211, 0.00211, 0.00844, 0.01688, 0.03376, 0.0844, 0.1688 mg/mL.

2.3.2 Preparation of Sample Solution

Take out 1.0g Flemingia philippinensis Merr. et Rolfe. After precisely weighing, put it into a 25mL measuring bottle to get methanol ultrasonic treatment for 30 minutes. Keep the solution till it gets the room temperature to dilute it with methanol to the scale. Shake it to take the supernatant which would be filtrated by 0.45 μm Microporous filter membrane. Take the filtrate as the sample solution.

2.4 System Applicability

The sample solution is injected according to the above mentioned chromatographic conditions. Record the chromatogram (**Figure 1**). It is shown that the theoretical plate number of chromatographic peaks of genistein is no less than 3000 and the chromatographic peaks of each reference substance are in good shape with the separation degree between each peak no less than 1.5.



A t/min B

Figure 1; HPLC Chromatogram of Flemingia philippinensis Merr. et Rolfe**.**

A. Reference substance B. Sample

2.5 Linear Relationship

Reference solution is precisely weighed and prepared to solutions with concentration respectively of 0.0000211, 0.00211, 0.0844, 0.01688, 0.03376, 0.0844, 0.1688 mg/mL. Samples are injected at 10μL and determined by HPLC. Record the peak area and draw the standard curve with peak area as ordinate and the sample volume as abscissa. Based on the regression equation of standard curve $Y=1.98953\times10^{-7}X$ and its correlation coefficient r=0.99997, the linear relationship performs well when the sample volume ranges from 0.0211 μg to 168.8 μg, which meets the experiment requirements.

2.6 Precision Test

Take reference solution of genistin, inject which for six times with HPLC to obtain the RSD as 0.325% each time.

2.7 Repeatability Test

Take samples from the same batch, determine which under the above mentioned chromatographic conditions for six time to obtain the RSD as 0.539%.

2.8 Sample Recovery Rate Test

Precisely weigh Flemingia philippinensis Merr. et Rolfe and divide them into nine samples (each sample about 0.5g) with three samples as a group. Respectively add reference substance precisely to determine with HPLC to calculate recovery rate. And the average recovery rate is 99.59% and RSD is 1.03%.

2.9 Determination of Sample Content

Take both different-place selected and marketed Flemingia philippinensis Merr. et Rolfe to determine with HPLC. Respectively inject sample solution and reference solution 10μL under the same chromatographic conditions to determine and record the chromatogram (**Figure 1**). Calculate the content of samples with external standard method. See **Table 1** for the results (n=2).

Sample Sources	Content of Genistin (%)		Average Content (%)	RSD(%)
	1	2		
Xinping (Roots)	0.0301	0.0300	0.0300	0.538
Yangbi (Roots)	0.00132	0.00131	0.00131	0.438

Jinghong (Roots	0.0118	0.0119	0.0119	0.597
Xinping (Stem)	0.0368	0.0369	0.0369	0.192
Xinping (Leaf)	0.0125	0.0126	0.0125	0.563
Marketed Sample 1	0.0629	0.0631	0.0630	0.224
Marketed Sample 2	0.0479	0.0469	0.0474	1.49
Marketed Sample 3	0.0577	0.0575	0.0576	0.246
Marketed Sample 4	0.0548	0.0544	0.0546	0.518

Table 1. HPLC Results of Flemingia philippinensis Merr. et Rolfe from Different Sources

3. Results and Discussion

HPLC is set up with genistin as the index to determine the content of three batches of field collected Flemingia philippinensis Merr. et Rolfe and four batches of marketed herbs. It is shown that the content of Flemingia philippinensis Merr. et Rolfe collected from Yangbi is the lowest (about 0.001%); while that collected from Xinping is the highest (0.03%). And the contents in the stems and leaves of Flemingia philippinensis Merr. et Rolfe collected in Xinping are respectively 0.037% and 0.012%. The content of marketed Flemingia philippinensis Merr. et Rolfe could reach as high as 0.063% or as low as 0.047%. Genistin is one of the flavonoids with higher content. It is of guiding significance of genistin for the quality control of medicine. However, this study indicates that the contents among collected and marketed Flemingia philippinensis Merr. et Rolfe varies significantly, so does that in different parts of the plant. Therefore, it should be further studied and discussed of the distribution and accumulation of content in different parts of the plant as well as the correlation among places of origin, collection seasons etc..

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